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Nerve Insensitivity Resistance to Pyrethroids in Heliothine Lepidoptera*

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Abstract: A neurophysiological assay developed previously was used to assess the incidence of nerve insensitivity resistance to synthetic pyrethroids in field strains of *Helicoverpa armigera*. Almost 70% of individuals from a sample of a highly pyrethroid-resistant population from Jiangsu Province, China were nerve-insensitive. Subsequent selection resulted in a strain homogeneous for expression of this mechanism. Likewise, over 95% of a sample from a strain of the insects from Andhra Pradesh, India were nerve-insensitive and a homogeneous strain was developed. Development of a nerve-insensitive laboratory strain of *Heliothis virescens* was undertaken but homozygosity could not be obtained. It is suggested that high fitness costs may be associated with this mechanism. The incidence of nerve insensitivity in Heliothine pests is reviewed and the role of phenotypic expression assays in molecular studies highlighted.

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1 INTRODUCTION

Resistance to the synthetic pyrethroid insecticides has developed in the cotton bollworm *Helicoverpa armigera* Hübn., throughout Asia, the Indian sub-continent and Australia.[1,2] Likewise, in the Americas pyrethroid resistance is common in the tobacco budworm *Heliothis virescens* (F.)[3] and becoming increasingly reported in the corn earworm *Helicoverpa zea* (Boddie).[4,5] The mechanisms of resistance have been extensively studied in both *H. armigera* and *H. virescens*, and include delayed penetration, nerve insensitivity and enhanced metabolism due to monooxygenases and to esterases.[6,7]

The target site for pyrethroid insecticides (and DDT) is the voltage-gated sodium channel in nerve membranes.[8,9] Insects in populations that have been repeatedly selected with pyrethroids frequently show a resistance to their knockdown effect. This has been presumed to involve modifications of the sodium channel which affect pyrethroid binding or to involve changes in gene expression. The involvement of a sodium channel modification in knockdown resistance was first suggested by electrophysiological studies which showed that the nervous system of *kdr* houseflies was less sensitive to the action of pyrethroids.[10]

For studies of pyrethroid resistance in heliothine caterpillars we developed a simple neurophysiological assay to detect nerve insensitivity resistance.[11,12] The assay has since been modified to detect nerve insensitivity in adults[14] and has also been used here with late-stage larvae. Initially, this assay was designed for screening populations for nerve insensitivity and for use in studies of the inheritance of nerve insensitivity. It is now being exploited as a means of associating phenotypic expression of nerve insensitivity with molecular modifications in the sodium channel. In this paper we

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TABLE 1
Effects of Exposure to *cis*-Cypermethrin on Spontaneous Neuronal Activity in the Cumulative Dose Response Assay in Nerve Preparations from Third-Instar Larvae of Susceptible and Pyrethroid-Resistant Strains of *Heliothis virescens* from USA

	Number of individuals responding in assay at: cis-*cypermethrin concentration* (*nM*)						
Strain	*n*	*1*	*5*	*10*	*50*	*100*	> 100
Susceptible laboratory strains							
BRC	30	23	7	0	0	0	0
B-94	5	3	1	1	0	0	0
SUR	10	6	1	1	2	0	0
Pyrethroid-resistant USA strains							
Saint Joseph, LA	30	4	6	6	1	3	12
Red River, LA	31	7	5	3	1	4	11

provide an up-to-date survey of the incidence of nerve insensitivity in Heliothine lepidoptera, particularly in those strains where molecular studies are under way.

2 EXPERIMENTAL METHODS

2.1 Insects

Samples of eggs and/or neonate larvae of a pyrethroid-resistant strain (JSFX) of *H. armigera* were collected randomly from cotton plants in the Fengxie county area of Jiangsu province in China in June 1994 and pupae shipped to Reading. F2 to F4 insects were selected at third-instar with diagnostic doses of up to 0·04 μg of fenvalerate per larva. Offspring of single pair crosses at F5 were screened with diagnostic doses of fenvalerate (0·04 μg per larva) following pre-treatment (30 min) with piperonyl butoxide (PB; 20 μg per larva). F6 families with high resistance to fenvalerate and low synergism by PB were selected and single pairs mated and selected for two further generations.

A random collection of *H. armigera* eggs was made from chickpea plots at ICRISAT Centre, Hyderabad, India during November 1996 and pupae shipped to the UK. A total of 100 single breeding pairs of adults was set up and allowed to oviposit. Following oviposition the adults were tested for the presence of nerve insensitivity using the neurophysiological assay. The offspring of nerve-insensitive parents were retained and the presence of nerve insensitivity reconfirmed at the third-instar.

The laboratory susceptible B-94 and LSU strains of *H. virescens* were supplied by Zeneca Agrochemicals, Jealott's Hill, UK and Louisiana State University, USA respectively. The susceptible SUR strain was developed from mass crosses between B-94 and LSU. The NIR strain was derived from a putative nerve-insensitive strain NI1 (Imong, pers.comm.) by back-crossing four times to the SUR strain. Each back-cross was interspersed with a test cross, the progeny of which were selected at first-instar using a discriminating concentration of cypermethrin (25 μg. ml^{-1}). To emphasise the ubiquitous nature of this resistance mechanism the results obtained with these *H. armigera* field strains were compared with field strains of *H. virescens* from Saint Joseph and Red River in Louisiana, USA as described elsewhere.[11,13]

2.2 Insecticides

Technical *cis*-cypermethrin (98·4%) and fenvalerate (97·5%) were supplied as technical materials by Zeneca Agrochemicals, Jealott's Hill, UK and Shell Research Limited, Sittingbourne, UK, respectively.

2.3 Neurophysiological assay for nerve insensitivity

Extracellular recordings of spontaneous neuronal activity in peripheral nerves exposed in partly dissected larvae or adults bathed in saline containing *cis*-cypermethrin were made at 25($\pm$ 3)°C using a cumulative dose response assay described in detail previously.[11,13] Briefly, third-instar larvae (19–24 mg) or adults (wings and legs removed) were opened dorsomedially and pinned out on a layer of Sylgard resin (Dow Corning, UK) and the ventral and lateral body walls exposed. Peripheral nerves were picked up using an insulated 27-gauge steel suction recording electrode. Spontaneous extracellular neuronal activity was amplified using a signal conditioning system (Neurolog, Digitimer, UK) before being relayed to a Maclab 2e data recording and analysis instrument connected to an Apple Macintosh LCII computer. Discrimination of action potentials from background noise was made using a visually adjusted threshold and activity was

recorded in 5-min periods. Following a minimum of two 5-min control periods in saline, the nerves were bathed in increasing concentrations of insecticide for successive 5-min periods. The end point of the assay was determined as the lowest concentration in which the frequency of action potential was over five times greater than the mean value recorded during the control period.

3 RESULTS

3.1 Neurophysiology

3.1.1 Heliothis virescens *susceptible strains*

In the neurophysiological assay, larval preparations of the BRC susceptible strain all responded to 5 nM or lower concentrations of *cis*-cypermethrin (Table 1). Whilst seven of the 10 preparations of the SUR strain responded at a similar level, the remaining three individuals responded to the next two concentrations. A small sample of B-94 strain larvae all responded at or below 10 nM *cis*-cypermethrin. The neurophysiological assay was also used to examine larger larvae and, in order to distinguish between susceptible and resistant preparations, an increased range of concentrations of the insecticide was required compared to that used in the third-instar assay. Fifth-instar preparations of the SUR strain responded to between 5 and 100 nM *cis*-cypermethrin with a peak at 25 nM (Table 2).

3.1.2 Heliothis virescens *USA pyrethroid-resistant strains*

Individual preparations from fifth-instar larvae of the selected NIR strain were examined using a modified neurophysiological assay and compared with the susceptible SUR stain. Around 45% of NIR larval strain preparations responded only at the highest concentration (1 μM) of *cis*-cypermethrin used in the fifth-instar assay or did not respond (Table 2). The remaining insects responded at similar concentrations to the SUR susceptible strain, confirming the heterogeneous nature of this strain. For comparison, Table 1 shows two pyrethroid-resistant field strains, Red River and St Joseph, which gave a range of responses in the neurophysiological assay. A bimodal distribution around the 50 nM concentration of *cis*-cypermethrin clearly separated resistant and susceptible responses (Table 1).

TABLE 2
Effects of Exposure to *cis*-Cypermethrin on Spontaneous Neuronal Activity in the Cumulative Dose Response Assay in Nerve Preparations from Fifth-Instar Larvae of Susceptible and Pyrethroid-Resistant Laboratory Strains of *Heliothis virescens*

		Number of individuals responding in assay at: cis-*cypermethrin concentration* (*nM*)					
Strain	*n*	*5*	*25*	*100*	*250*	*1000*	> 1000
Susceptible laboratory strain							
SUR	26	6	13	7	0	0	0
Pyrethroid-resistant laboratory strain							
NIR	33	5	7	3	3	1	11

TABLE 3
Effects of Exposure to *cis*-Cypermethrin on Spontaneous Neuronal Activity in the Cumulative Dose Response Assay in Nerve Preparations from Third-Instar Larvae of Susceptible and Pyrethroid-Resistant Strains of *Helicoverpa amigera* from China and India

		Number of individuals responding in assay at: cis-*cypermethrin concentration* (*nM*)					
Strain	*n*	*1*	*5*	*10*	*50*	*100*	> 100
Susceptible laboratory strain							
Reading	44	21	15	7	1	0	0
Reading (series 2)	20	11	8	1	0	0	0
Pyrethroid-resistant China strains							
JSFX	26	1	3	4	4	5	9
JSFX, F6, family 43	26	0	0	1	4	7	14
JSFX, F9, family 37	49	0	0	0	0	10	39
JSFX, F9, fam. 37 (series 2)	20	0	0	0	0	7	13
Pyrethroid-resistant India strains							
Icrisat 96, P adults	30	0	0	1	0	4	25
Icrisat 96, F1, family 18	15	0	2	0	0	0	13
Icrisat 96, F1, family 48	45	0	0	0	1	1	13

3.1.3 Helicoverpa armigera *susceptible strain*
Forty-four third-instar larvae of the Reading susceptible strain were examined using the cumulative dose-response assay. All but one of these preparations (98%) responded with a greater than five-fold increase in spontaneous neuronal activity over that seen during the control period following addition of 1, 5 or 10 nM *cis*-cypermethrin (Table 3). In a second series, in which a further 20 preparations were tested, 95% of individuals responded at concentrations of *cis*-cypermethrin of 5 nM or less.

3.1.4 Helicoverpa armigera *China pyrethroid-resistant strains*
Over 68% of individual preparations from the JSFX strain (F5) of *H. armigera* only responded in the assay to concentrations of 50 nM *cis*-cypermethrin or above, or were immune to pyrethroid action (Table 3). The neurophysiological responses of individual preparations from the selected F6 family 43 were confined to concentrations of 10 nM and above and over 80% responded only at or above the highest dose (100 nM). All individual preparations of the highly resistant selected F9 family 37 responded only at or above 100 nM *cis*-cypermethrin, confirming this to be a strain homozygous for nerve insensitivity.

3.1.5 Heliothis armigera *India pyrethroid-resistant strains*
Adults of the Icrisat 96 strain were tested neurophysiologically directly following emergence from field-collected pupae shipped to the UK. Over 95% of these individuals responded only at the highest concentration of *cis*-cypermethrin used in the assay or did not respond at all (Table 3). Prior to the neurophysiological assay, eggs were collected from these adults and the third-instar larvae from two families of the following generation examined. In both these resistant families, over 86% of individuals failed to respond to concentrations of *cis*-cypermethrin up to 100 nM, indicating a high frequency of nerve-insensitive individuals.

4 DISCUSSION

Neurophysiologically detectable nerve insensitivity resistance to pyrethroids has been documented in samples of *H. armigera* from Thailand,[14] Australia[6] and India,[15] *H. virescens* from the USA[7,11,12] and most recently *H. zea* from the USA.[5] A feature of all these field collections is that they are heterogeneous with respect to the incidence of nerve insensitivity, ranging from barely detectable, as with *H. zea*, through being modestly present in a great many collections to very frequent in a small minority of samples. In the majority of cases of resistance to pyrethroids in *H. armigera*, nerve insensitivity is present along with a more powerful metabolic resistance mechanism.[1,2,16] In Australia there are indications that nerve insensitivity might have declined in importance.[6]

Strains of *H. virescens* from the USA were frequently found to include a range of phenotypes with respect to nerve insensitivity. The Red River and Saint Joseph strains described here are examples of collections from areas of Louisiana where pyrethroid resistance was prevalent[3] and both strains include a high proportion of nerve-insensitive individuals together with susceptible insects. It is presumed that responses at or above 50 nM are typical of resistant insects, whilst responses at or below 10 nM are typical of susceptible insects. Significantly, at the time of collection, metabolic resistance was rare in these populations.[11]

For a comparison of the SUR and NIR laboratory strains of *H. virescens*, the neurophysiological assay was modified to examine fifth-instar insects. The reasons for the marked increases in concentration required to elicit responses in both susceptible and resistant fifth-instar larval preparations over those required for third-instar larvae are unclear but may relate to the vastly greater fat deposits in the bodies of fifth-instar compared to third-instar larvae. Nevertheless, the assay distinguishes nerve-sensitive and nerve-insensitive individuals and emphasises the heterogeneous nature of the NIR strain. Recent studies have indicated a strong fitness cost associated with nerve insensitivity in this strain[17] and this is a likely reason for the difficulties involved in the preparation of isogenic lines. In view of this, it is clearly essential to test individual insects neurophysiologically to confirm phenotypic expression of nerve insensitivity prior to preparation of samples for molecular analysis of sodium channel genes.

Recent collections of *H. armigera* from India were examined as adults without interposing a generation of laboratory breeding. A high frequency of expression of nerve insensitivity resistance in this collection was indicated by the high percentage of individuals which responded only at or above the highest concentration used in the assay. The offspring from these parents were likewise highly nerve-insensitive and samples of these recently obtained larvae are being examined for sodium channel mutations which might be associated with resistance. Recent studies by Kranthi *et al.*[18] indicate the presence of both enhanced monooxygenase and esterase activity in temporally variable proportions in *H. armigera* from the Nagpur area of Maharashtra state in India and infer that a large proportion of the residual resistance which remains unsynergised by metabolic inhibitors may be due to nerve insensitivity, although definitive evidence for this has yet to be obtained. A similar interpretation has been applied to the data generated by the pyrethoid resistance monitoring scheme in Australia,[1] where nerve insensitivity has been detected

previously, although there are indications that its importance has now declined.[6]

Resistance to pyrethroids in *H. armigera* is widespread in China[19,20] and insects of a strain collected from Jiangsu Province were over 200-fold resistant to fenvalerate before laboratory selection. Although this resistance was largely synergisable by PB and by 1,2,4-trichloro-3-(2-propynyloxy)benzene (TCPB; data not shown), over 68% of individuals tested neurophysiologically were also nerve-insensitive. Following selection, during which resistance to fenvalerate was increased 18-fold, the strain was shown to be homogeneous for nerve insensitivity with all 59 individuals tested responding at or above 100 nM *cis*-cypermethrin. In view of the strong expression of nerve insensitivity in what appears to be a homogeneous strain, these insects are being analysed for the presence of sodium channel gene mutations.

A number of recent studies have demonstrated a genetic linkage between nerve insensitivity resistance and *para* (*Drosophila melanogaster* Meig.) homologous sodium channel genes in the housefly (*Musca domestica* L.),[21] *H. virescens*[22] and the German cockroach (*Blattella germanica* L.).[23] A mutation encoding a *Leu* to *Phe* change in transmembrane segment six of homology domain II (IIS6) of the housefly *para* homologous sodium channel gene has recently been shown to be associated with this resistance in *kdr* strains.[24] In *super-kdr* strains, a second mutation in the cytoplasmic linker between S4 and S5 involving a *Met* to *Thr* substitution has also been noted. Interestingly, in *B. germanica*,[25,26] the *Leu* to *Phe* change has also been observed at the homologous position to that reported for the *kdr* substitution in the housefly. Recently, a *Leu* to *His* substitution, again in the homologous position, has been found associated with pyrethroid resistance in *H. virescens*.[27] Not all individuals examined showed this, implying either that more than one sodium channel mutation may be contributing to pyrethroid resistance in field populations or that other resistance mechanisms are present. It is clear that the use of a simple phenotypic assay for nerve insensitivity is essential in studies which attempt to link resistance genotypes with their expression.

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